ORAC Assay for the determination of antioxidant capacity in foods

Measuring oxygen radical absorbance capacity with the Infinite® 200 PRO multimode reader

Introduction

Reactive oxygen species (ROS) are generated as natural by-products of the cellular metabolism. They are involved in various biological processes, functioning as important signal mediators. However, excess intracellular levels of ROS may result in cell and tissue damage, and are associated with degenerative diseases, most notably cancer. In healthy individuals, intracellular antioxidant systems maintain ROS levels below a critical threshold, permitting essential ROS-mediated signaling processes to function, but preventing ROS overproduction and potential tissue damage [1]. Cells that fail to compensate and neutralize heightened ROS levels die by apoptosis to avoid passing on ROS-caused DNA damage to daughter cells. Any dysfunctions in the cellular antioxidant systems can therefore have serious consequences. In addition to the cells' own antioxidant systems, various studies have suggested a relationship between an antioxidant-rich diet and a good health status, implicating that the consumption of antioxidant-containing foods can help to maintain health and even prevent certain diseases [2].

A well-established and reliable method to determine the antioxidant capacity of a substance is the oxygen radical absorbance capacity (ORAC) assay [3]. It is based on the inhibition of oxyradical-induced oxidation of 2,2'-azobis-(2-methylpropionamidine) dihydrochloride (AAPH) by substances with antioxidant properties. Peroxyl radicals produced in a time-dependent manner during the thermal decomposition of AAPH will quench the fluorescence signal. In the presence of a substance with antioxidant properties the fluorescence reduction is inhibited, depending on the substance's ORAC capacity. The dynamics of the signal inhibition, expressed as the area under the curve (AUC), are used to quantify the antioxidant capacity, expressed as the ORAC value, by comparing the sample AUC to an antioxidant standard curve generated with Trolox, a water-soluble vitamin E analog. This application note describes the use of the Infinite® F200 PRO in combination with a commercially available ORAC assay kit, using different beverages as antioxidant samples.

Materials and Methods

- Black 96-well microplates (Greiner Bio-One)
- Black 384-well microplates (Greiner Bio-One)
- OxiSelect™ ORAC Activity Assay (Cell Biolabs, Inc.)
- Phosphate buffered saline (PBS)
- Infinite F200 PRO

The ORAC values of different beverages were determined in the Infinite F200 PRO, using the OxiSelect ORAC Activity Assay by Cell Biolabs, Inc [4]. The following beverages were tested: apple juice, blackcurrant juice, grapefruit juice, orange juice, pineapple juice, red grape juice and red wine. The fruit juices and red wine were obtained from a local supermarket. Care was taken to purchase only 100 % pure juices.

The OxiSelect assay was performed according to the manufacturer's instructions [4]. The standard curve was generated by diluting Trolox from 50-3.1 µM in PBS.

	96-well plate	384-well plate
Fluorescein [10 nM]	150 µl	75 µl
Trolox or sample	25 µl	12.5 µl
PBS	25 µl	12.5 µl
Total volume/well	**200 µl**	**100 µl**

Table 1 Assay reagent mix in 96- and 384-well plates.

The assay plates were covered with sealing film to minimize evaporation and incubated for 30 min at 37°C using the instrument's incubation function.

Fluorescence measurements were taken every 60 seconds (see instrument settings summarized in Table 2). After the first three cycles (representing the baseline signal), AAPH was injected into each well to initiate the ROS generation.

Measurement mode	**Fluorescence Intensity Top**
Excitation wavelength	485 (20) nm
Emission wavelength	535 (25) nm
Kinetic cycles	60-100
Kinetic interval	60 sec
Kinetic condition	Inject AAPH after cycle 3: 96-well: 25 µl 384-well: 12.5 µl
Flash number	10
Gain	optimal
Lag time	0 µs
Integration time	20 µs

Table 2 Instrument settings for ORAC assay.

Results

An antioxidant standard curve was generated using different concentrations of Trolox. Figure 1 shows the dose-dependent loss of fluorescence induced by the addition of AAPH. The measured fluorescence values were normalized to 100 %, with 100 % reflecting the initial fluorescence signals after the addition of AAPH.

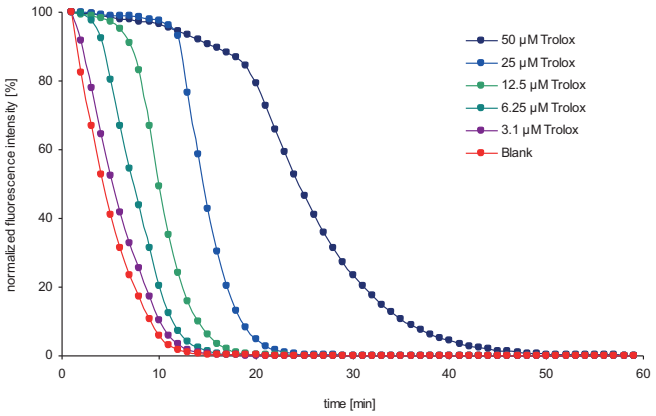

Figure 1 Signal curves measured for different Trolox concentrations (results normalized to initial fluorescence signals after AAPH addition).

The antioxidant capacity, expressed as the AUC, was calculated as follows:

$$AUC = 1 + RFU_1/RFU_0 + RFU_2/RFU_0 + RFU_3/RFU_0 + \ldots\ldots + RFU_n/RFU_0$$

RFU_0 = relative fluorescence units at time point zero
RFU_n = relative fluorescence units at time points

The Net AUC values, reflecting the blank-corrected AUC values for each standard, were plotted against the Trolox concentrations, and the linearity of the standard curve was calculated (Figure 2).

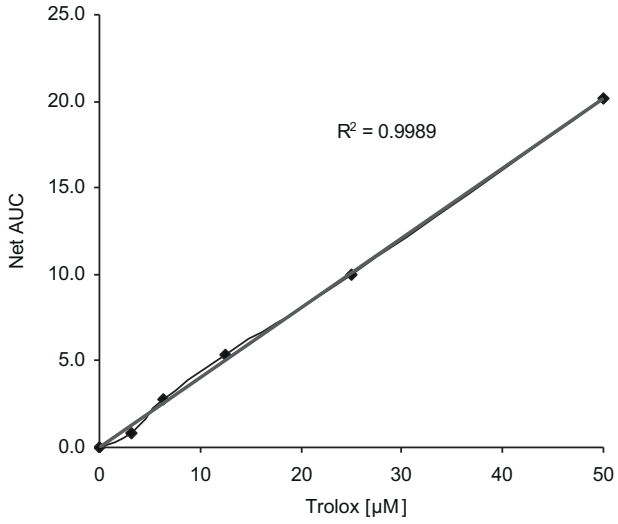

Figure 2 Trolox standard curve.

During the analysis of the beverage samples, it became evident that some samples could not be analyzed in an undiluted state, because they reduced the fluorescence signal even in the absence of the ROS-generating agent (AAPH). The individual fluorescence quenching potential was assessed for all samples using dilutions from 1:1 to 1:1000. The impact on the fluorescence signal for two example beverages is shown in Figure 3. In both cases, significant signal quenching was observed with undiluted fluids. This effect was also apparent in the 1:2 and 1:10 dilutions, and gradually disappeared when the samples were diluted further.

Therefore, 1:100 dilutions of the beverages were used in all further analyses. All fruit juice dilutions were freshly prepared prior to each experiment. Results measured in 96- and 384-well plates were similar; the results shown in the following section were obtained in 96-well plates.

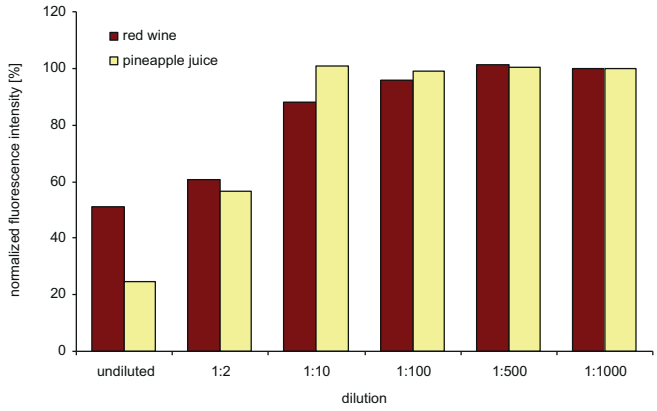

Figure 3 Fluorescence quenching by fruit juices at different dilutions.

As shown in Figure 4, the beverages analyzed exhibited different signal kinetics and antioxidant capacities. The highest antioxidant potential was seen with red wine. Among the non-alcoholic beverages, blackcurrant and grapefruit juices were the most potent antioxidants, while orange and pineapple juices showed the least antioxidant potential.

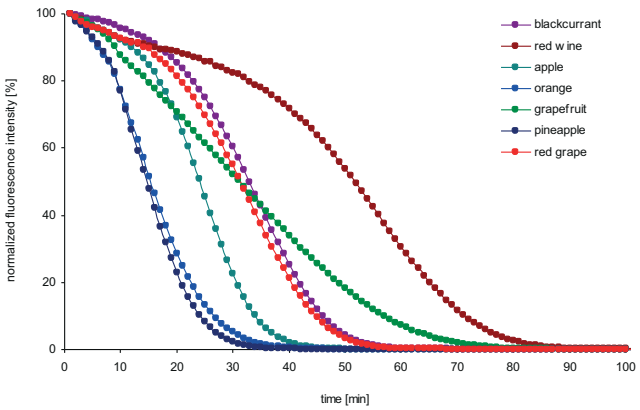

Figure 4 Signal curves for different beverage samples (all used in 1:100 dilution).

The ORAC values of the samples can be extrapolated from the Trolox standard curve. Table 3 summarizes the calculated ORAC values of the samples tested.

Sample	ORAC value
Blackcurrant	9.7
Red wine	15.9
Apple	6.4
Orange	3.5
Grapefruit	9.7
Pineapple	3.1
Red grape	9.0

Table 3 ORAC values of beverages tested (µmol TE/ml).

Importantly, the ORAC values determined for the beverages are only valid for the products tested. Depending on the fruit content and composition, the ORAC values for beverages from other manufacturers may differ.

Conclusion

The results summarized in this application note demonstrate the excellent applicability of the Infinite F200 PRO for the ORAC assay in 96- and 384-well plates (with adapted volumes per well). The instrument's on-board injector system and the incubation function greatly facilitate the assay, making the Infinite F200 PRO ideally equipped for reliable and reproducible ORAC assays.

Literature

(1) Oberdanner, C. ROS and *Antioxidant Systems in Apoptosis.* VDM Verlag, 2008. ISBN 978-3836482110

(2) Slavin JL et al. *Health benefits of fruits and vegetables*. Adv Nutr. 2012. 1;3(4):506-16

(3) Cao G et al. *Oxygen-radical absorbance capacity assay for antioxidants.* Free Radical Biol. Med. 1993. 14: 303-311.

(4) *OxiSelect™ Oxygen Radical Antioxidant Capacity (ORAC) Activity Assay*. Kit instructions. STA-345. Cell Biolabs Inc.

List of Abbreviations

AAPH	2,2'-azobis-(2-methylpropionamidine) dihydrochloride
AUC	Area under curve
ORAC	Oxygen radical antioxidant capacity
PBS	Phosphate buffered saline
RFU	Relative fluorescence units
ROS	Reactive oxygen species
TE	Trolox equivalent

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Australia +61 3 9647 4111 **Austria** +43 62 46 89 33 **Belgium** +32 15 42 13 19 **China** +86 21 2206 3206 **Denmark** +45 70 23 44 50 **France** +33 4 72 76 04 80
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